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II. REMARKS

Formal Matters

Claims 6-8 and 15-24 are pending.

Claims 6-8 and 15-19 were examined. Claim 19 is allowed and claims 6-8 and 15-18 stand rejected.

Claims 6 and 15 are amended and claims 20-24 are new. The amendments to the claims were made solely in the interest of expediting prosecution, and are not to be construed as an acquiescence to any objection or rejection of any claim. Support for the amendments and the new claims is found in the claims as originally filed and throughout the specification, in particular at the following exemplary locations: page 3, line 25; page 5, lines 1-15; page 2, line 5; page 10, line 30; page 15, lines 10-18, Figs. 5, 6 and 7. Accordingly, no new matter is added.

Claims 1-5 and 9-12 are canceled without prejudice to renewal.

The Applicants respectfully request reconsideration of the application in view of the remarks made herein.

Rejection under 35 U.S.C § 103

Claims 6-8 and 15-18 stand rejected under 35 U.S.C § 103 as assertedly obvious over Kourilsky (Biochimie (1977) 53:1111-1114) in view of Brenner (USPN 5,604,097). Specifically, the Office asserts one of skill in the art would combine Kourilsky's urea-based hybridization methods with Brenner's oligonucleotide array methods to provide the claimed invention. The Applicants respectfully traverse the rejection.

According to MPEP §2142 and current law, to establish a *prima facie* case of obviousness, three basic criteria must be met. *Graham v. John Deere*, 383 U.S. 1, 148 USPQ 459 (1966). First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. *In re Fine*, 5 USPQ2d 1596 (Fed. Cir. 1988); *In re Jones,* 21 USPQ2d 1941 (Fed. Cir. 1992). Second, there must be a reasonable expectation of success. *In re Merck & Co., Inc.*, 231 USPQ 375 (Fed. Cir. 1986). Finally, the prior art reference, or references when combined, must teach or suggest all the claim limitations. *In re Royka*, 180 USPQ 580 (CCPA 1974). All three criteria must be met. If all three criteria are not met, a *prima facie* case of obviousness has not been established.

Further, as set forth in MPEP §716.02, a rejection based on obviousness may be withdrawn upon a showing of unexpected results.

In view of MPEP §2142 and current law, the Applicants respectfully assert that the claimed subject matter is non-obvious over Kourilsky and Brenner. Factual support for the Applicants assertions are set forth below.

The cited combination of references do not suggest the claimed invention

The claims stand rejected over a combination of Kourilsky (disclosing urea-based hybridization methods using DNA from phage lambda (λ)) and Brenner (disclosing an oligonucleotide array). Neither Kourilsky nor Brenner suggest combining their respective teachings to produce the subject matter of the rejected claims. In fact, neither Kourilsky nor Brenner recognize any problem that can be solved by lowering the hybridization temperature of an oligonucleotide array assay. Accordingly, on their face, the cited references themselves provide no suggestion to lower the hybridization temperature of an oligonucleotide array assay.

The Office argues that one of skill in the art would have been motivated to use glass as a solid support in Kourilsky's DNA-DNA hybridization methods to provide an automatic system. However, the mere substitution of a glass support into Kourilsky's method does not provide the method being claimed: Kourilsky's polynucleotides contains *intact* DNA from phage λ (see page 1111; beginning of paragraph entitled "Preparation of DNA filters"), not oligonucleotides, as required by the rejected claims. Further, the rejected claims also recite a microarray. Substituting a glass support into Kourilsky's method provides neither a method involving oligonucleotides nor a method involving a microarray, let alone a method involving an oligonucleotide microarray.

Accordingly, this rejection, in its current form, fails to provide any motivation to provide the claimed invention.

Claims could not be practiced with any reasonable expectation of success

Even if the Office could find evidence that one of skill in the art would be motivated to combine the teachings of Kourilsky and Brenner to provide the claimed invention, the Applicants respectfully submit that one of skill in the art would not produce the claimed method with any reasonable expectation of success.

The rejected claims as well as the new claims are directed to methods that produce *specific* binding between a target nucleic acid and an *oligonucleotide* probe that is present in a *microarray*.

The Applicants respectfully assert that the urea-based teachings of Kourilsky cannot be readily transferred into methods involving an oligonucleotide microarray to provide specific binding. Factual support for the Applicants position is set forth below.

Firstly, one of skill in the art would have no reasonable expectation that the combination of Kourilsky and Brenner would provide a method to produce *specific* hybridization between an oligonucleotide probe and a target nucleic acid, as required by the instant claims. The question of binding specificity is crucial to oligonucleotide microarray assays since oligonucleotide microarrays contain a plurality of different oligonucleotides that are each contacted with a single sample under the same conditions. In other words, non-specific binding of a target to oligonucleotide probes other than the oligonucleotide probe intended is extremely undesirable in microarray experiments. Accordingly, one of skill in the art would not practice the claimed methods with any reasonable expectation of success if he thought that binding would be anything other than specific.

Neither Kourilsky nor Brenner discusses specificity of binding between an oligonucleotide and a labeled target, or, for that matter, any two nucleic acids, in the presence of urea. One of skill in the art, therefore, could not extrapolate the teachings of Kourilsky or Brenner to provide any method that provided a reasonable expectation of specific binding between an oligonucleotide to a target nucleic acid. In fact, as discussed in response to the previous Office Action, Kourilsky explicitly expresses doubt that his methods could be used to provide specific binding to other polynucleotides. The Examiner's attention is again drawn to paragraph 2 of Kourilsky's discussion, in which Kourilsky states "...**conditions optimal for the hybridization of certain fragments may, in fact, be selective against other fragments**" and "**In certain instances, the specificity of the reaction may be questionable** [17]. **Therefore the above conditions do not necessarily apply to other experimental circumstances**, and may have to be modified accordingly". (Emphasis added). In view of Kourilsky's doubt, one of skill in the art would have no expectation that Kourilsky's findings could readily be adapted to a method that provides specific binding to an oligonucleotide.

Furthermore, the Office argues that Kourilsky does not specifically point out that his doubt is directed to hybridization of oligonucleotides. Nevertheless, the Applicants

respectfully submit that at the time of filing of the instant application, one of skill in the art would have been well aware that specific hybridization conditions for Kourilsky's polynucleotide and the claim-recited oligonucleotides are very different, and, accordingly, that urea would have an unpredictable effect on the specificity of oligonucleotide hybridization.

Further evidencing this assertion are sections from two frequently used laboratory manuals, usually referred to as "Maniatis" and "Ausubel" (see Exhibits A and B). This factual evidence has been discussed in previous communications with the Office and is not discussed herein in great detail solely in the interest of brevity. The Applicants respectfully submit that in view of this evidence, a skilled person would have no expectation that urea, which is cited as being previously been used as a hybridization temperature reducing agent only for *phage lambda* DNA molecules (as taught by Kourilsky), would be effective as a hybridization temperature reducing agent for *oligonucleotides* (as recited in the claims). Moreover, in view of the teachings of Ausubel and Maniatis, one of skill in the art would not be able to predict the effect of urea on the *specificity* of binding of a oligonucleotide, much less that urea could be used to effect specific binding of a target and an oligonucleotide a reduced temperature.

The claimed method provide unexpected results

Finally, the claimed urea-based methods provide enhanced results which could not have been predicted from the teachings of the cited art, in comparison to equivalent methods using other denaturants. As evidence of these advantages, Applicants have attached herewith a post-filing publication demonstrating the unexpected benefits of the presently claimed methods and compositions. The date appearing on this publication is "October 2002", well after the filing date of the instant application.

This publication, which is a product literature brochure from MWG Biotech AG (submitted herewith as Exhibit C), describes hybridizing a microarray of oligonucleotide probes with target nucleic acids from rat liver and kidney, in a variety of different denaturants, including salt (e.g., SSC and SSPE), two different concentrations of formamide, and urea. The results from this experiment are shown on page 2 of the publication in the graph entitled Fig. 1. Quoting from the publication: "The slide-to-slide correlation of ratios were clearly better in urea-buffer (see Figure 2)."

These data demonstrate that urea buffers are superior to buffers containing other denaturants (even those buffers containing salt) in oligonucleotide microarray experiments. This superiority could not have been predicted by the teachings of Kourilsky or Brenner Accordingly, one of skill in the art could not have predicted the success of the presently claimed invention.

Summary

In asserting that the claimed methods are non-obvious over the combined teachings of Kourilsky and Brenner, the Applicants have provided factual evidence that one of skill in the art would find no motivation to combine the teachings of Kourilsky and Brenner to provide the claimed invention with any expectation of success. Further, the Applicants have provided a factual showing of unexpected results that should be sufficient, according to the MPEP §716.02 and current law, for withdrawal of this rejection. Merely stating that it is "common logic that shorter DNA would be more easily hybridized to other DNA strand in the presence of urea ...." (see paragraph 2 of Office Action) cannot over-ride this factual support for non-obviousness of the claimed methods.

The Applicants respectfully submit that this rejection has been adequately addressed. In view of the foregoing discussion, the Applicants respectfully request withdrawal of this rejection.

CONCLUSION

The Applicants submit that all of the claims are in condition for allowance, which action is requested. If the Examiner finds that a telephone conference would expedite the prosecution of this application, the Examiner is invited to telephone Timothy Joyce at (650) 485 4310.

The Commissioner is hereby authorized to charge any fees under 37 C.F.R. §§1.16 and 1.17 that may be required by this paper, or to credit any overpayment, to Deposit Account No. 50-1078.

Respectfully submitted,
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APP EXHIBIT A

concentrations of protein are believed to interfere with the annealing of the probe to its target. This quenching of the hybridization signal is particularly noticeable when oligonucleotides or probes less than 100 nucleotides in length are used.

7. In the presence of 10% dextran sulfate or 10% polyethylene glycol, the rate of hybridization is accelerated approximately tenfold (Wahl et al. 1979; Renz and Kurz 1984; Amasino 1986) because nucleic acids are excluded from the volume of the solution occupied by the polymer and their effective concentration is therefore increased. Although dextran sulfate and polyethylene glycol are useful in circumstances where the rate of hybridization is the limiting factor in detecting rare sequences (e.g., northern or genomic Southern blots), they are of no benefit when screening bacterial colonies or bacterial plaques. In addition, they can sometimes lead to high backgrounds, and hybridization solutions containing them are always difficult to handle because of their viscosity. We therefore recommend that dextran sulfate and polyethylene glycol not be used unless the rate of hybridization is very slow, the filter contains very small amounts of DNA, or the amount of radiolabeled probe is limiting.

8. To maximize the rate of annealing of the probe with its target, hybridizations are usually carried out in solutions of high ionic strength (6 × SSC or 6 × SSPE) at a temperature that is 20–25°C below the melting temperature (T_m). Both solutions work equally well when hybridization is carried out in aqueous solvents. However, when formamide is included in the hybridization buffer, 6 × SSPE is preferred because of its greater buffering power.

9. In general, the washing conditions should be as stringent as possible (i.e., a combination of temperature and salt concentration should be chosen that is approximately 12–20°C below the calculated T_m of the hybrid under study). The temperature and salt conditions can often be determined empirically in preliminary experiments in which samples of genomic DNA immobilized on filters are hybridized to the probe of interest and then washed under conditions of different stringencies.

10. To minimize background problems, it is best to hybridize for the shortest possible time using the minimum amount of probe. For Southern hybridization of mammalian genomic DNA where each lane of the gel contains 10 μg of DNA, 10–20 ng/ml radiolabeled probe (sp. act. = 10^9 cpm/μg or greater) should be used and hybridization should be carried out for 12–16 hours at 68°C in aqueous solution or for 24 hours at 42°C in 50% formamide. For Southern hybridization of fragments of cloned DNA where each band of the restriction digest contains 10 ng of DNA or more, much less probe is required. Typically, hybridization is carried out for 6–8 hours using 1–2 ng/ml radiolabeled probe (sp. act. = 10^9 cpm/μg or greater).

11. *Useful facts*:

    a. The T_m of the hybrid formed between the probe and its target may be estimated from the following equation (Bolton and McCarthy 1962):

$$T_m = 81.5°C + 16.6(\log_{10}[Na^+]) + 0.41(\text{fraction G + C}) - 0.63(\%\ \text{formamide}) - (600/l)$$

where l = the length of the hybrid in base pairs.

This equation is valid for:

- Concentrations of Na^+ in the range of 0.01 M to 0.4 M. It predicts T_m less accurately in solutions of higher $[Na^+]$.
- DNAs whose G + C content is in the range of 30% to 75%. Note that the depression of T_m in solutions containing formamide is greater for poly(dA:dT) (0.75°C/1% formamide) and less for DNAs rich in poly(dG:dC) (0.50°C/1% formamide) (Casey and Davidson 1977).

The equation applies to the "reversible" T_m that is defined by optical measurement of hyperchromicity at OD_{257}. The "irreversible" T_m, which is more important for autoradiographic detection of DNA hybrids, is usually 7–10°C higher than that predicted by the equation.

Similar equations have been derived for:

i. RNA probes hybridizing to immobilized RNA (Bodkin and Knudson 1985)

$$T_m = 79.8°C + 18.5(\log_{10}[Na^+]) + 0.58(\text{fraction G + C}) + 11.8(\text{fraction G + C})^2 - 0.35(\%\ \text{formamide}) - (820/l)$$

ii. DNA:RNA hybrids (Casey and Davidson 1977)

$$T_m = 79.8°C + 18.5(\log_{10}[Na^+]) + 0.58(\text{fraction G + C}) + 11.8(\text{fraction G + C})^2 - 0.50(\%\ \text{formamide}) - (820/l)$$

Comparison of these equations shows that the relative stability of nucleic acid hybrids decreases in the following order: RNA:RNA (most stable), RNA:DNA (less stable), and DNA:DNA (least stable). In aqueous solutions, the T_m of a DNA:DNA hybrid is approximately 10°C lower than that of the equivalent RNA:RNA hybrid. In 80% formamide, the T_m of an RNA:DNA hybrid is approximately 10°C higher than that of the equivalent DNA:DNA hybrid.

b. The T_m of a double-stranded DNA decreases by 1–1.5°C with every 1% decrease in homology (Bonner et al. 1973).

The above equations apply only to hybrids greater than 100 nucleotides in length. The behavior of oligonucleotide probes is described in detail in Chapter 11.

For a general discussion of hybridization of nucleic acids bound to solid supports, see Meinkoth and Wahl (1984).

EXHIBIT 13

UNIT 6.3

Using DNA Fragments as Probes

BASIC PROTOCOL

HYBRIDIZATION IN FORMAMIDE

Materials (see APPENDIX 1 for items with ✓)

Nitrocellulose membrane filters bearing plaques, colonies, or DNA (*UNITS 6.1 & 6.2*)
✓ Hybridization solution I
Radiolabeled probe, 1 to 15 ng/ml (*UNIT 3.5*)
✓ 2 mg/ml sonicated herring sperm DNA
✓ Low-stringency wash buffer I
✓ High-stringency wash buffer I, prewarmed

1. Wet filters in turn with 5 to 20 ml hybridization solution I, producing a stack of 10 to 20 filters. Transfer to a sealable bag and add enough hybridization solution to cover. Seal and prehybridize ≥1 hr at 42°C.

   No more than ten 20 × 20–cm square filters or twenty 82-mm discs should be placed in one stack.

2. Boil radioactive probe (1 to 15 ng/ml hybridization reaction at $>5 \times 10^7$ cpm/mg) with 2 mg (1 ml) sonicated herring sperm DNA for 10 min in a screw-cap tube.

3. Transfer to ice and add 2 ml hybridization solution I. Add probe mixture via syringe with 18-G needle to filters, reseal, mix thoroughly, and incubate overnight at 42°C.

4. Rinse filters three times with 500 ml low-stringency wash buffer I, room temperature, 10 to 15 min for each rinse.

   CAUTION: *The solution is extremely radioactive; handle carefully.*

5. Rinse filters twice with 500 ml high-stringency wash buffer I (prewarmed to wash temperature), 15 to 20 min for each rinse.

   Determine wash temperature empirically. If homology between probe and target approaches 100%, use a high-temperature wash of 65° to 75°C. For low homology and short probe lengths, lower temperature to 37° to 40°C. Wash very short probes <100 bp at lower temperatures regardless of homology.

6. Mount filters wet wrapped in plastic wrap or dry on plastic backing (e.g., used X-ray film) for autoradiography.

ALTERNATE PROTOCOL

HYBRIDIZATION IN AQUEOUS SOLUTION

Additional Materials (see APPENDIX 1 for items with ✓)

✓ Hybridization solution II
✓ Low-stringency wash buffer II
✓ High-stringency wash buffer II

1. Prehybridize as in Basic Protocol except incubate in hybridization solution II at 65°C.

Prepare probe as in Basic Protocol and dilute with 2 ml hybridization solution II. Hybridize overnight at 65°C. Remove hybridization solution and rinse twice with low-stringency wash buffer

Wash filters quickly 5 to 8 times with high-stringency wash buffer II at 65°C. Leave in final wash ~20 min. Washed filters should produce a nonspecific signal only a few-fold above background levels.

References: Church and Gilbert, 1984; Denhardt, 1966.

Contributor: William M. Strauss

UNIT 6.4

Using Synthetic Oligonucleotides as Probes

BASIC PROTOCOL 1

HYBRIDIZATION IN SODIUM CHLORIDE/SODIUM CITRATE (SSC)

Materials (see APPENDIX 1 for items with ✓)

Membrane filters bearing plasmid, bacteriophage, or cosmid libraries (*UNITS 6.1 & 6.2*)
3× SSC/0.1% SDS
✓ Prehybridization solution
✓ SSC hybridization solution
6× SSC/0.05% sodium pyrophosphate, prewarmed

1. Prepare duplicate nitrocellulose filters (processed and baked) of bacterial colonies or bacteriophage plaques. Wash 82-mm filters 3 to 5 times in 500 ml 3× SSC/0.1% SDS (50 filters) at room temperature. Then wash at 65°C at least 1.5 hr to overnight.

   Use filter forceps (without serrated tips) to handle the membrane filters.

2. Prehybridize in prehybridization solution 1 hr at 37°C.

3. Transfer up to 20 filters into sealable bags containing ≥20 ml SSC hybridization solution, and add 0.125 ng (for bacterial colonies) to 1.0 ng (for bacteriophage plaques) of each ^{32}P-labeled oligonucleotide/ml hybridization solution in one bag. Hybridize oligonucleotides 14 to 48 hr at the temperatures indicated:

   14-base—room temperature
   17-base—37°C
   20-base—42°C
   23-base—48°C.

4. Remove filters and wash three to five times in 6× SSC/0.05% pyrophosphate 5 to 15 min at room temperature. Wash 30 min in prewarmed 6× SSC/0.05% pyrophosphate at the temperatures indicated:

   14-base—37°C
   17-base—48°C
   20-base—55°C
   23-base—60°C.

10/001,688 Exhibit C

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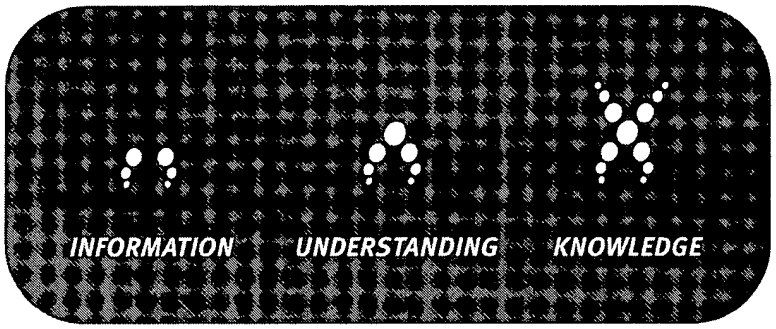
THE DEVELOPMENT OF A HYBRIDIZATION BUFFER FOR LARGE-VOLUME HYBRIDIZATIONS



MWGd
GENOMIC DIAGNOSIS
Always a Result ahead.

: October 2002

The complete automation of the hybridization process including sample preparation is an important long-term goal for allowing high throughput in terms of maximum reproducibility and quality assurance. This study focuses on the hybridization process itself. In order to allow hybridization in large volumes (70-350µl) – as it is necessary for most automated hybridization chambers –, we had to develop and optimize such a system. Moreover, hybridization in a larger volume is supposed to generate more reproducible results. Consequently we found that primarily the development of a hybridization buffer allowing stringent and sensitive, reproducible hybridizations was necessary.

As a test system, the MWG Rat5K Array was used and hybridized competitively using rat tissue (liver against kidney). This array contains 5535 Oligos (50 mers) representing ORFs, 100 replicated spots and 125 Arabidopsis control oligos. Hybridization samples were generated by direct labeling and amplification. The hybridizations were carried out using MWG Gene Frames specially designed for MWG arrays in order to simulate the large volume approach. Using these frames a MWG Rat 5K Array is hybridized in 240µl probe volume.

Denaturing agents

A hybridization buffer can be a pure salt based buffer. Different salts are possible, the most common are SSC and SSPE. Their concentration depends on the favored stringency. Formamide is also frequently added because of its strong denaturing features, although it may cause problems for large volume hybridizations. Alternatives to formamide would be urea, which is much less toxic, and guanidine-hydrochloride, a chaotrophic salt, often used in DNA-kits. This study focused on the comparison of formamide and urea. A 50% formamide-buffer hybridized under a coverslip was used as a reference. Figure 1 shows a profile display comparing the ratios derived from experiments using different denaturing agents in the hybridization buffer. The results show that ratios were compressed in pure salt-based buffer, whereas lowering the formamide concentration to 30% yielded comparable results to the reference. In urea containing buffer ratios were even spread under the same hybridization conditions.

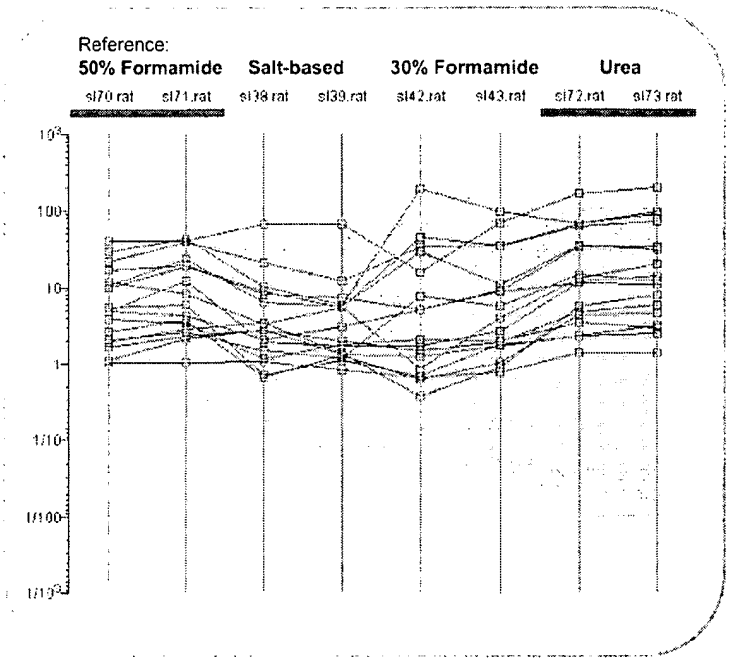


Figure 1. Denaturing agents: Profile Display. This figure shows a profile display comparing the ratios derived from experiments using different denaturing agents in the hybridization buffer. The highlighted genes represent a selection which is expressed in liver. The first two slides were hybridized under coverslips using a 50% formamide buffer (reference approach).

The slide-to-slide correlation of ratios were clearly better in urea-buffer (see Figure 2). Here, 98.9% of the values varied within a twofold range. These were only 89.7% in 30% formamide-buffer. However, signal intensities were significantly lower with both buffers than with the reference approach.

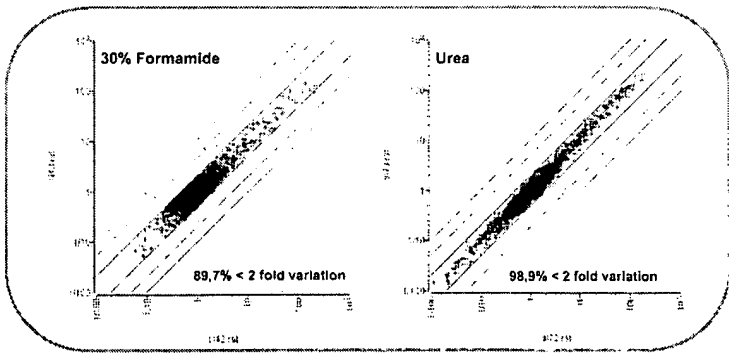


Figure 2. Denaturing agents: Slide-to-slide correlation. Log/log blots expressing the slide-to-slide correlation of ratios in buffers containing 30% formamide and urea, respectively. Ratios varying within a twofold range are marked red.

Signal enhancement

Thus, in order to combine the specificity of buffers containing denaturing agents with sensitivity, the next step was to focus on signal enhancement strategies. The addition of dextran sulfate for instance, results in a virtual volume reduction effect. In aqueous solutions it is strongly hydrated. Therefore macromolecules have no access to the hydrating water which causes an apparent increase in probe concentration and consequently higher hybridization rates. Another possibility would be the decrease of the concentration of denaturing agents. As this results in reduced stringency, the concentration has to be adapted carefully. In order to increase diffusion of the sample over the array, the assay can be agitated during the incubation time. The easiest possibility is the incubation in a shaking waterbath. The effect of the addition of 5% dextran sulfate is shown in Figure 3 in direct comparison to an array hybridized without it.

The addition obviously resulted in a very strong signal increase because of the volume reduction effect. An increased background and gradient formation were also frequently observed. Moreover, the specificity was significantly decreased. Therefore this approach was rejected.

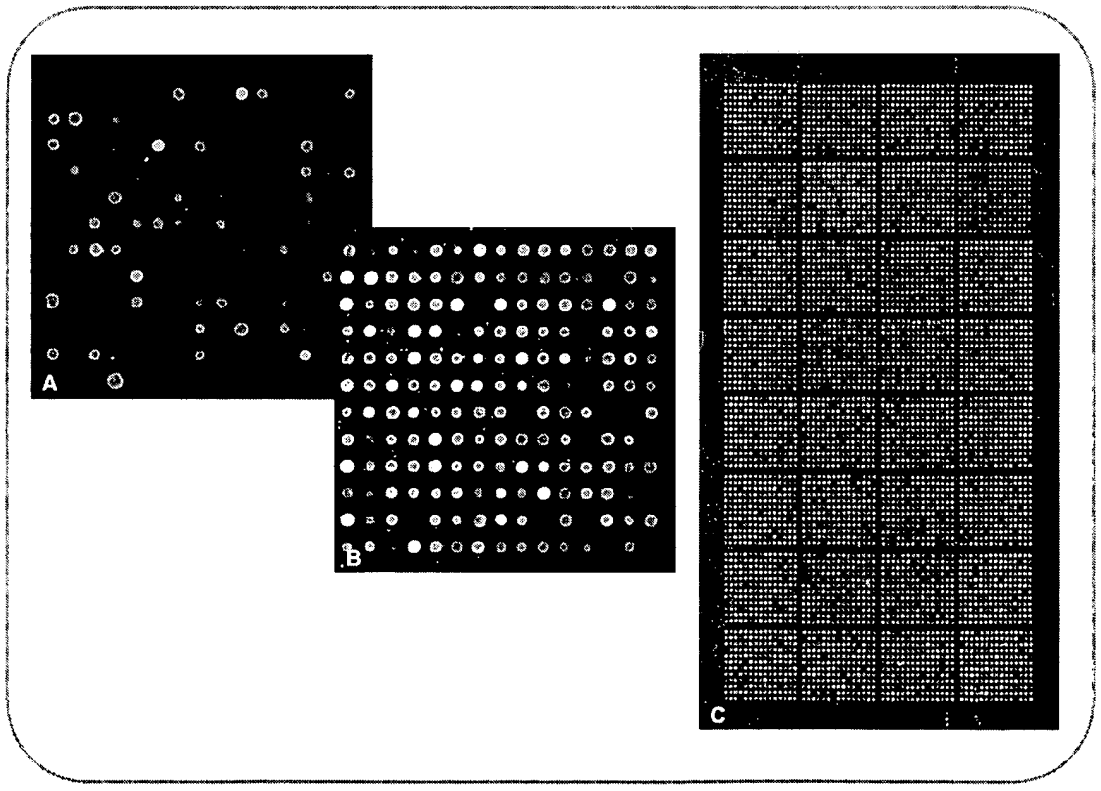


Figure 3. Signal enhancement: Dextran sulfate. This figure shows the effect of the addition of 5% dextran sulfate (B, C) in direct comparison to an array hybridized without it (A).

A decreasing concentration of formamide from 30% to 10% led to significantly increased signals (three-/fourfold) as shown in Figure 4.

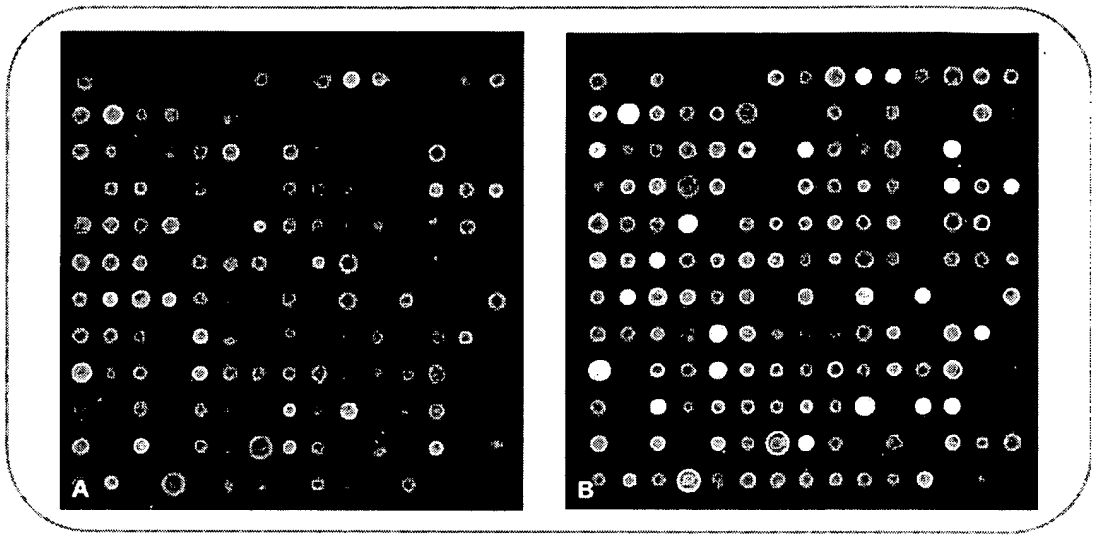


Figure 4. Signal enhancement: Formamide concentration. This figure shows the effect of a decreasing concentration of formamide from 30% (A) to 10% (B).

However, as shown in the profile display of the ratios derived from these two experiments (see Figure 5), the specificity was also decreased. Due to the significant loss of specificity, this step was also not successful.

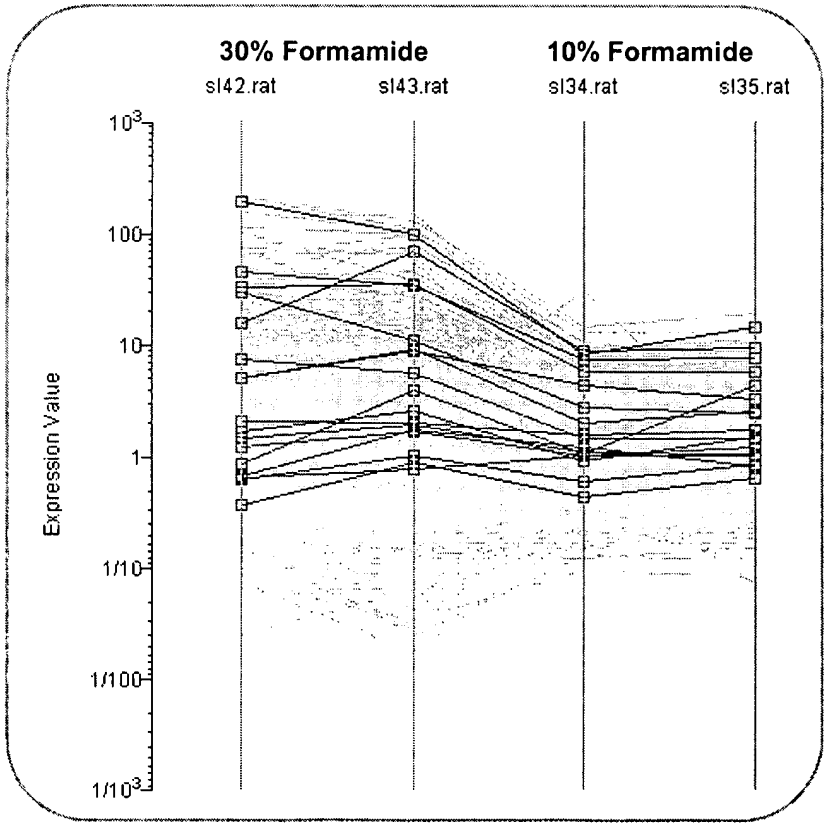


Figure 5. Signal enhancement: Formamide concentration. This figure shows a profile display of the ratios derived from the two experiments reducing the formamide concentration. The highlighted genes represent a selection which is expressed in liver.

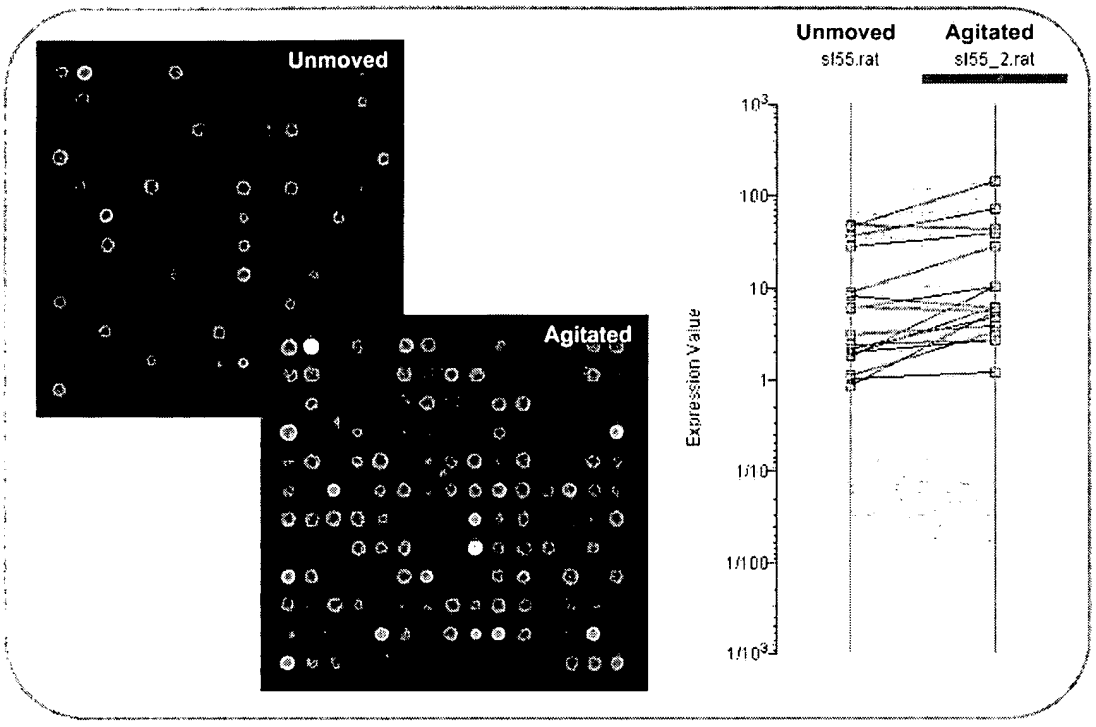


Figure 6. Signal enhancement: Agitation. This figure shows the influence of agitated hybridization by incubation in a shaking waterbath on signal intensities (left) and ratios (right). The highlighted genes represent a selection which is expressed in liver.

Meanwhile, agitation by incubation in a shaking waterbath also led to signal enhancement as shown in Figure 6 in direct comparison of the two slides. Thereby, the specificity was also slightly increased because of the enhanced diffusion (profile display of the ratios in Figure 6). In total, the increase of signal intensity was less strong than that achieved by lowering the formamide concentration. But as the specificity was even positively affected, this approach was favored.

Stringent conditions

An important factor during the improvement of the hybridization process are the hybridization conditions and their respective stringency. First the hybridization temperature was varied in a range of 42 up to 55°C. Increasing temperature generally leads to increased stringency but background problems on the slides also tend to occur the higher the temperature is chosen. The increase of wash temperature has the same impact. Second, the salt concentrations in hybridization and wash buffers can be lowered, but the influence on stringency is not that significant than those of the other adjustments. Another point as already mentioned, is agitation during the hybridization process. This has a positive influence on stringency and will moreover enhance signal intensities.

Strategy and Solution

The strategy for developing a specific and sensitive hybridization buffer can thus be

- to use a pure salt-based buffer, or
- to add denaturing agents.

In the first case a careful adjustment of hybridization conditions (hybridization and wash temperature, salt concentration in hybridization and wash buffer) would be necessary to achieve an optimal balance between specificity and sensitivity. If denaturing agents are used, their concentration has to be carefully adjusted. The results of our study clearly show that the second option was most effective. Thus the solution in our case was a hybridization buffer containing a denaturing agent, precisely an urea-based buffer, because of the lower toxicity and better slide-to-slide reproducibility compared to formamide. In addition agitation during incubation appeared to be recommended.

Conclusion and perspective

In conclusion it can be stated that a hybridization system was created in this study which allows a good imitation of automated hybridization. Moreover, future automation of the hybridization process

- will reduce variations due to manual handling, and thus further increase reproducibility
- and will probably also increase signal intensities and significantly reduce hybridization times due to the agitation of the hybridization solution.

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